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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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TEST METHODS AND DEVICES

Field of the Invention

This invention relates to test methods and devices, and more particularly to methods for differentiating between states of an analyte that exists as various forms, eg isoforms.

Background to the Invention

Tests are available, or have been proposed, which purport to provide clinically significant information about hormonal levels of relevance to the menopause. The principal hormone of interest is follicle stimulating hormone (FSH). The post-menopausal state has been associated with a rise in the level of circulating FSH. For this purpose tests have been developed to detect the level of FSH in body fluid samples such as blood and urine. These tests are intended to detect "total" FSH, in the sense that they do not discriminate between different isoforms of FSH.

These known tests are used by clinicians in recommending and monitoring hormone replacement therapy (HRT). As the menopause is also associated with a drop in the level of circulating estrogen metabolites, HRT normally involves administration of estrogen in order to reduce this deficit and counteract symptoms associated with the menopause.

Although it is known that FSH exists in various forms, the clinical significance of these in relation to conditions such as the menopause is poorly understood. The differing forms may be isoforms or glycoforms. However, the existence

of these differing forms calls into question the soundness of "total" FSH tests as a basis for good clinical diagnosis.

There is a need for an improved method of monitoring gonadotrophin hormones, especially FSH, to provide more reliable diagnosis of menopausal conditions and to facilitate the prescription and regulation of HRT.

More generally, there is a need for a method for differentiating between states of an analyte that exists as a plurality of forms, especially when the nature and/or relative amounts of such forms present in a sample of the analyte may be of clinical significance. The forms may differ from one another in either physical characteristics (eg "isoforms" separable by charge) or chemical characteristics (eg "glycoforms" in the case of FSH or similar molecules), or indeed both.

General Description of the Invention

The invention provides a method for differentiating between two states of an analyte that exists in a plurality of forms, which states differ from one another in the nature and/or amount of one or more forms present therein, in which method a sample, or contemporaneous samples, containing the analyte are subjected to a heterogeneous sandwich-format specific binding assay utilising a labelled first binding agent specific for the analyte and an unlabelled second binding agent specific for the analyte to provide a first test signal proportional to the amount of analyte present in the sample, and is also subjected to a homogeneous sandwich-format specific binding assay utilising the same pair of labelled and unlabelled analyte-specific binding agents to provide a second test signal proportional to the amount of

analyte present in the test sample, at least one member of said pair of binding agents having a different specificity for each of said two states of said analyte, and the first test signal is compared to the second test signal.

Preferably, each member of said pair of binding agents has a different specificity for each of said two states of said analyte.

Conveniently, a combined test result may be expressed as a ratio of the two test signals. Optionally, the ratio of the two test signals is compared to a standard ratio for one or other of the two states to determine in which state the sample analyte exists.

The method of the invention is especially applicable when the analyte is a gonadotrophin, such as FSH.

Preferably each binding agent is an antibody, especially a monoclonal antibody.

In one embodiment, in the heterogeneous assay a sample is incubated with a solid phase on which is immobilised the unlabelled binding reagent, and thereafter following a step to remove unbound analyte the solid phase is incubated with the labelled binding reagent.

In the homogeneous assay a sample is simultaneously incubated with a solid phase on which the unlabelled binding agent is immobilised and with the labelled binding agent in solution or suspension. Preferably, in the homogeneous assay the sample is simultaneously incubated with the unlabelled binding agent in solution or suspension and with the labelled binding agent in solution or suspension, and

the unlabelled binding agent is thereafter immobilised on a solid phase.

As one option, immobilisation of the unlabelled binding agent on the solid phase is effected through a specific binding reaction, such as an avidin-biotin interaction.

In particular the invention provides a method of monitoring the hormonal status of an individual human female subject in which the contemporaneous tests are conducted repeatedly, ie. At regular intervals such as every few weeks, to determine whether the gonadotrophin level and its character are changing in a manner which indicates entry into or departure from a menopausal state.

Another embodiment of the invention is an assay device for testing a body fluid sample obtained from a human female, the device having a first gonadotrophin-responsive signal-producing means that provides a readable signal by means of a heterogeneous assay as described herein, and a second gonadotrophin-responsive signal-producing means that provides a readable signal by means of a homogeneous assay as described herein, the assay signals differing depending on whether the sample is derived from a pre-menopausal or post-menopausal subject.

Each readable signal can be caused by the binding in a detection zone of a specific binding agent labelled with a direct particulate label, such as a gold sol or coloured latex particle. Alternatively, other signal-producing labels can be used, for example enzyme labels, fluorescent labels or radio-labels.

The contemporaneous tests of the invention can be conducted repeatedly, generally at an interval of at least a week, to monitor the effectiveness of a course of HRT.

Although FSH is the preferred analyte for use in accordance with the invention, other members of the gonadotrophin family can be used. These include human chorionic gonadotrophin (hCG), luteinizing hormone (LH) and thyroid stimulating hormone (TSH). All of these gonadotrophins are glycopeptides. Their principal structure comprises two peptide chains. One peptide chain, known as the alpha chain, is common to all members of the family. The other peptide change, known as the beta chain, differs in each molecule. In addition, each molecule contains glycoprotein side chains. The detailed structure of these molecules is not completely understood. However it is believed that variations in the glycoprotein side chains give rise to different forms of each molecule. Thus, in the case of FSH for example, on present scientific knowledge it is believed that the alpha and beta peptide chains are the same in all FSH forms, but subtle differences occur in the glycoprotein side chains. It is believed that the relative proportions of the forms of FSH existing in the menopause state are different from those in the pre-menopause state.

Prior to this invention it was not appreciated that a specific binding assay could be developed which would differentiate between the FSH forms, to an extent sufficient to enable worthwhile detection of a menopausal state to be achieved, or that by using two different formats together an assay could provide enhanced differentiation.

In a preferred embodiment of the invention both assays are of the sandwich format. Each assay therefore requires two

specific antibodies, one preferably directed against the alpha chain and the other preferably against the beta chain of the FSH molecule. The antibody pairs must be different. In a preferred embodiment the invention uses two sandwich-format immunoassays assays for FSH, one heterogeneous and the other homogeneous, in which the antibodies are directed against the alpha and beta peptide chains of the molecule, but are exhibiting differences in specificity for FSH caused by subtle changes in the glycoprotein side chains.

Antibody pairs appropriate for use in the invention can be identified by screening a range of anti-FSH antibody pairs against FSH samples obtained from pre-menopausal and post menopausal women.

In order to provide a source of antibodies from which to select an antibody pair which under the circumstances of the method of the invention differentiate between analyte forms, it is desirable, although not essential, to raise a panel of antibodies against the analyte forms in question. This can be done by routine hybridoma technology.

A particular aspect of the invention in relation to its application to the analysis of FSH samples is a pair of novel anti-FSH monoclonal antibodies that distinguish between pre-menopausal and post-menopausal FSH samples. Two murine hybridoma cell lines each expressing one of these novel monoclonal antibodies have been deposited in accordance with the provisions of the Budapest Treaty 1977 in the European Collection of Cell Cultures (ECACC) as follows:

a) Balb/c murine hybridoma clone "4813.2" expressing an anti-beta-FSH monoclonal antibody: ECACC 00032004.

c) Balb/c murine hybridoma clone "4882.1" expressing an anti-alpha-FSH monoclonal antibody: ECACC 00032005.

The invention includes the use of either or both of the anti-FSH monoclonal antibodies as expressed by these deposited cell lines, in a method or analytical test device as set forth herein.

In practice the two assays should be performed on the same clinical sample, sub-divided if necessary, or on two samples obtained from the same individual subject at more or less the same time, so that the two assays give results that can fairly be compared with each other. It is in this sense that we regard the assays as being contemporaneous. The results of the two contemporaneous assays are compared to determine whether a menopausal state exists.

In one embodiment the test results can be interpreted on a qualitative or semi-quantitative basis, for example by eye if the two assays give rise to visible test readings which can be interpreted readily, for example through differences in colour intensity. If necessary this visual determination can be aided by the provision of a reference standard. The two assays can be configured to aid visual assessment.

For more accurate diagnosis of menopausal conditions it may be appropriate for the assay results to be determined numerically. This will usually require a sophisticated reading system, such as by optical transmission or reflectance and which is amenable to measuring small changes in signal intensity and relating these to FSH concentrations. In this situation it may be appropriate to determine the numerical ratio of the signals of the first

and second assays. A significant change in this ratio can indicate transition from a pre-menopausal to a post-menopausal state, or vice-versa. Thus the results from a series of double tests performed, for example, every few weeks, can be collated and any change in the observed signal ratio used to diagnose a change in condition.

For the purposes of HRT monitoring, the HRT treatment, either in terms of the therapeutic product used or its dose level, can be modulated to maintain the ratio value from successive double tests at a pre-determined level, for example.

Test devices using the assays of the invention can be provided for home use or for use in clinics or doctor's offices. Alternatively laboratory-style assays can be used. Preferred assay formats involve the single step format as described, for example, in EP-A-291194. These assays can be used if desired in combination with an electronic reader, for example as described in WO 95/13531. In this instance preferably the electronic reader has an information downloading facility, eg. by means of a transferable datacard ("smart card"), from which a user, eg clinician, can transfer data to a computer during consultation with the patient, in order that stored information from repeated tests can be interpreted properly for diagnostic purposes. The computer can include programmed information that assists the clinician in establishing an appropriate HRT treatment for the individual subject.

Generally, the method of the invention involves the use of one pair of specific binding agents in two contemporaneous assays differing in format.

The following example illustrates aspects of the invention in greater detail.

Example

1. Raising anti-FSH monoclonal antibodies

Balb/c mice were immunised with human FSH preparations, derived from urine and pituitary sources, purified by immuno-affinity prior to immunisation. Monoclonal antibodies were produced from the immunised mice using conventional cloning techniques, by fusing spleen cells with SP2/Ag14 cells as the immortal partner.

The subunit specificity of the anti-FSH monoclonal antibodies were assigned by means of anti-alpha subunit antibodies cross reactive with LH, TSH and hCG.

2. Identification of anti-FSH antibody pairs with fertile state bias

a) A panel of seven human urine samples were used to screen various antibody pairs. The samples from young fertile women (under 35 years of age with regular menstrual cycles) were taken at the (1)early follicular, (2)mid-follicular, (3)ovulation, (4)mid-luteal and (5)late luteal phases of menstrual cycles. For each fertile phase pooled samples from two individuals were used. The phase of the menstrual cycle was determined retrospectively by the urinary profiles of the hormones FSH,LH, E3G and P3G. In addition to the fertile samples, two post menopausal urine samples were used (6) 1 month before commencement of HRT treatment and (7)one taken at least one month after HRT treatment began.

For use in the antibody screen the urine panel was normalised based on FSH concentration estimates. The FSH concentration estimates were obtained using commercially available anti-FSH monoclonal antibodies (Clone No's. 6601 and 6602 from Medix) in a sandwich-format ELISA assay. Samples were normalised by being concentrated using centrifuge filtration.

b) Screening procedure

1) FSH antibodies for screening were prepared to concentrations of 2.5µg/ml in 0.2M Sodium Carbonate buffer pH 8.0.

2) 200µl of the antibody dilutions were added to wells in High binding Greiner 96-well microtitre plates, which were then incubated overnight at 37°C.

3) The plates were washed three times in PBSTA.

4) 100µl of 0.38M Tris was added to into all of the plate wells, except the blanking wells to which PBSTA was added. 100µl of each urine sample from the screening panel (see paragraph a) were added at 15.3mIU/ml (based on the 6602/6601 assay estimates) to triplicate wells for each antibody/conjugate pairing.

5) The plates were incubated for 1 hour at room temperature.

6) Step 3 was repeated.

7) 200µl of an optimum dilution in PBSTA of alkaline-phosphatase conjugated anti-beta FSH subunit antibody was added to wells sensitised with anti-alpha FSH subunit capture antibodies. 200µl of an optimum dilution in PBSTA of alkaline-phosphatase conjugated anti-alpha FSH subunit antibody was added to wells sensitised with anti-beta FSH subunit capture antibodies.

8) As a control in each screening run the urine panel was tested with a reference assay using Medix clone No. 6601 conjugate paired with Medix clone No.6602 as the capture antibody.

9) Step 5 was repeated.

10) Step 3 was repeated.

11) 200µl of DEAE substrate was pipetted into all wells of all plates.

12) Step 5 was repeated.

13) The plates were read on a Dynatech plate reader at 405nm after 1hour 30 minutes incubation.

14) The mean value of the triplicates were then calculated, and compared to the Medix assay O.D. values. This allowed antibody pairs showing bias in sample recognition relative to the Medix assay to be identified.

The two hydridoma cell lines referred to earlier, now deposited with the ECACC, were selected using this procedure.

3. Use of heterogeneous/homogeneous FSH assays for menopause confirmation

The method described in section 4 below was used to test 8 consecutive daily urine samples (around mid-cycle) taken from one fertile woman, and 9 consecutive daily urine samples from one post-menopausal woman.

The FSH concentration in each sample was measured by the heterogeneous and homogeneous assays. The ratios obtained are shown in the Table below, and clearly differentiate between fertile and post-menopausal states.

4. Test methods

Both assays were carried out in a "Delfia" [RTM] Time Resolved Fluoroimmunoassay, supplied by Perkin Elmer Life Sciences, using the standard reagents and buffers as supplied, except where indicated.

a) Heterogeneous assay

1) FluoroNunc 96-well microtitre plates were sensitised with 200µl of 5µg.ml 4813 antibody in a coating buffer overnight at 4°C. The coating buffer contained:

0.01M phosphate pH 7.2
0.9% sodium chloride
0.02% sodium azide

2) 4813-sensitised plates were washed 2 times with Wash Concentrate.

3) 200µl of a blocking buffer was added to each well, and incubated for 1 hour at room temperature with shaking. The blocking buffer was the coating buffer plus 2% BSA.

4) Step 2 was repeated.

5) 50µl of FSH standard or urine sample and 200µul Assay Buffer was added per well.

6) Incubated for 6 hours at room temperature with shaking.

7) Wash 3 times with Wash Concentrate.

8) 200µl of Europium-labelled 4882 antibody in Assay Buffer added, and incubated for 1 hour at room temperature with shaking. The labelling was conducted using a commercial Europium labelling kit from Perkin Elmer.

9) Step 7 was repeated.

10) 200µl Enhancement Solution added to each well.

11) Incubation for 5 minutes at room temperature with shaking.

12) Read result.

b) Homogeneous assay

1) Proceed through steps (1) to (4) of assay (a).

2) Add 200µl FSH standard or urine sample, and 200 Eu-labelled 4882 antibody, and incubate for 2 hours at room temperature with shaking.

3) Proceed through steps (9) to (12) of assay (a).

Individual and Sample Day	Result Ratio of Heterogeneous to Homogeneous Assays
Fertile	
D14	3.5
D15	3.8
D16	3.8
D17	4.0
D18	3.8
D19	2.9
D20	3.4
D21	3.1
Post-Menopausal	
D10	1.2
D11	1.3
D12	1.2
D13	1.8
D14	1.3
D16	1.3
D17	1.3
D18	1.3
D19	1.5

CLAIMS

1. A method for differentiating between two states of an analyte that exists in a plurality of forms, which states differ from one another in the nature and/or amount of one or more forms present therein, in which method a sample or contemperaneous samples containing the analyte are subjected to a heterogeneous sandwich-format specific binding assay utilising a labelled first binding agent specific for the analyte and an unlabelled second binding agent specific for the analyte to provide a first test signal proportional to the amount of analyte present in the sample, and are also subjected to a homogeneous sandwich-format specific binding assay utilising the same pair of labelled and unlabelled analyte-specific binding agents to provide a second test signal proportional to the amount of analyte present in the test sample, at least one member of said pair of binding agents having a different specificity for each of said two states of said analyte, and the first test signal is compared to the second test signal.

2. A method according to claim 1, wherein each member of said pair of binding agents has a different specificity for each of said two states of said analyte.

3. A method according to claim 1 or claim 2, wherein a combined test result is expressed as a ratio of the two test signals.

4. A method according to claim 3, wherein the ratio of the two test signals is compared to a standard ratio for one or other of the two states to determine in which state the sample analyte exists.

5. A method according to any one of the preceding claims, wherein the analyte is a gonadotrophin.

6. A method according to claim 5, wherein the analyte is follicle stimulating hormone (FSH).

7. A method according to any one of the preceding claims, wherein each binding agent is an antibody.

8. A method according to claim 7, wherein each binding agent is a monoclonal antibody.

9. A method according to any one of the preceding claims, wherein in the heterogeneous assay the sample is incubated with a solid phase on which is immobilised the unlabelled binding reagent, and thereafter following a step to remove unbound analyte the solid phase is incubated with the labelled binding reagent.

10. A method according to any one of the preceding claims, wherein in the homogeneous assay a sample is simultaneously incubated with a solid phase on which the unlabelled binding agent is immobilised and with the labelled binding agent in solution or suspension.

11. A method according to claim 10, wherein in the homogeneous assay a sample is simultaneously incubated with the unlabelled binding agent in solution or suspension and with the labelled binding agent in

solution or suspension, and the unlabelled binding agent is thereafter immobilised on a solid phase.

12. A method according to claim 11, wherein immobilisation of the unlabelled binding agent on the solid phase is effected through a specific binding reaction.

13. A method according to claim 12, wherein the specific binding reaction is an avidin-biotin interaction.

14. A method for differentiating between an FSH sample indicative of a present or impending fertile status of the human ovulation cycle and an FSH sample indicative of a present or impending infertile status of the human ovulation cycle, substantially as hereinbefore described.

15. An anti-FSH monoclonal antibody as expressed by hybridoma cell line ECACC 00032004.

16. An anti-FSH monoclonal antibody as expressed by hybridoma cell line ECACC 00032005.

17. A method according to any one of claims 1 to 13, wherein the labelled binding agent is an antibody as claimed in claim 15 and the unlabelled binding agent is an antibody as claimed in claim 16.

18. A test device embodying a method as claimed in any one of the preceding claims.

Abstract

A method and test device for differentiating between states of an analyte that can exist in different forms, such as follicle stimulating hormone (FSH). The method or test device uses a pair of specific binding agents, especially monoclonal antibodies, in two assays for the same analyte. The assays, applied to contemporaneous samples, differ from one another in format, one being a heterogeneous sandwich-format assay and the other being homogeneous. A novel pair of anti-FSH monoclonal antibodies that can be used together in two such assays to differentiate pre-menopausal and post-menopausal FSH samples are disclosed.